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TITLE: The Effect of COX-2 Inhibitors on the Aromatase Gene (CYP19) Expression in Human Breast Cancer

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14. ABSTRACT
Aromatase (CYP19) is responsible for estrogen biosynthesis, and CYP-19 and cyclooxygenase-2 (COX-2) are both overexpressed in human breast cancers. Prostaglandin activates the CYP19 promotor and increases gene expression therefore we hypothesized that celecoxib, a selective COX-2 inhibitor, will decrease PG and decrease the expression of CYP19. To test this hypothesis, celecoxib was administered to breast cancer patients after the initial core biopsy tumor tissue and then tumor tissue was collected at the definitive surgery on OSU protocol 0125. A total of 8 breast cancer patients were enrolled until December 20, 2004 when the cardiovascular risks associated with celecoxib were made public. Accrual was suspended and the restarted in July 2005 with a revised protocol and consent form describing the cardiovascular risks. The trial was closed November 13, 2006 due to lack of accrual. There were no adverse events recorded during celecoxib administration. With only 8/34 (23%) of patients enrolled, there have no analysis of gene expression of CYP19. This trial failed to accrue, in part, over concerns about the cardiovascular risks.

15. SUBJECT TERMS
COX-2 inhibitors, CYP-19

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Introduction

Aromatase (CYP19) is responsible for estrogen biosynthesis, and CYP19 and cyclooxygenase-2 (COX-2) are both overexpressed in human breast cancers. Prostaglandin E_2 (PG) activates the CYP19 promotor and increases gene expression. Therefore, we hypothesized that celecoxib, a selective COX-2 inhibitor, would decrease PGE_2 and decrease the expression of CYP19. OSU protocol 0125 (Appendix A) was designed to test this hypothesis. In OSU 0125 women with newly diagnosed breast cancer received celecoxib 400 mg oral twice daily in the 10-14 day interval between initial diagnostic core biopsy and the definitive surgery (either lumpectomy or mastectomy). Fresh frozen and paraffin tumor tissues samples were collected before and after treatment with celecoxib. The sample size was 34 women. The primary endpoint was to evaluate gene expression of CYP19 by reverse transcriptase polymerase chain reaction (RT-PCR); secondary endpoints were to evaluate by immunohistochemical (IHC) the following: ER, progesterone receptor (PR), Her-2/neu, Ki-67, COX-1, COX-2, CYP19, and CD31. If our hypothesis is correct, then expression of the CYP19 gene will decrease in response to celecoxib. This pilot study will provide preliminary data to support a mechanism whereby COX-2 inhibitors decrease estrogen production within breast tumors by decreasing CYP19 expression.

Body

From 9/2/03 until 4/19/05, 13 women had their initial core biopsies collected for research purposes 8 of them enrolled on OSU 0125. Dr. Shapiro, the principal investigator, suspended the trial to further accrual on 12/20/04 in response to action letters received from the Clinical Investigations Branch (NCI) and Pfizer concerning celecoxib and increased cardiovascular risks. In accordance with the guidelines issued by the National Institute of Health, the protocol and consent form were amended to include information about the increased cardiovascular risks related to celecoxib. The OSU Cancer IRB approved this protocol amendment on February 7, 2005 as did the Army HSRRB on July 28, 2005. Because accrual to the study was suspended for about 6 months, Dr Shapiro requested a no-cost extension for this award. Subsequently, approval of the no-cost extension on June 27, 2005 was for 18 months. Trial was closed to further accrual November 13, 2006 because of lack of accrual.

Of the 5 women who had the initial core biopsies but didn't enroll on OSU 0125 after December 2004, 1 was ineligible because of taking another non-steroidal anti-inflammatory; 1 was determined to need neoadjuvant chemotherapy; 1 had a benign core biopsy, and 2 refused treatment with celecoxib because of concerns of toxicity. Despite having surgical principal investigator, Dr. Stephen Povoski, repeatedly discussing poor accrual at our monthly Breast Disease Specific Meeting, and having a dedicated research nurse available to discuss the trial literally at anytime, the trial failed to enroll subsequent breast cancer patients.

Key Findings

Eight patients have had core biopsies, were treated with celecoxib, and definitive breast surgery (Tables 1, 2). The median age was 56 years (range 42-73 years); 6 patients were stage II and 2 were stage I; 4 patients were hormone receptor positive, 4 were hormone receptor negative, and 4 were HER2 overexpressing. There were neither adverse events related to celecoxib nor any voluntary withdrawal of consent during the study period. With only 8/34 (23%) patients enrolled, the primary endpoints (gene expression of CYP19, ER by RT-PCR) and secondary endpoints (IHC for the following: ER, PR, Her-2/neu, Ki-67, COX-1, COX-2, CYP19, and CD31) were not been performed. These samples remain in storage.

Table 1

Core biopsy

Pt #	Date	Age (yr)	ER (%)	PR (%)	HER2	Histology	Grade
01	8/22/03	73	60	5	Negative*	IDC	3
02	9/24/03	47	> 70	40-50	3+	IDC	3
03	10/29/03	42	0	0	3+	IDC	3
04	2/20/04	56	< 1	< 1	3+	IDC	3
05	4/5/04	69	> 75	30	Negative*	ILC	2
06	8/03/04	65	0	0	0	IDC	3
07	10/14/04	68	0	0	3+	IDC	3
08	11/09/04	48	100	100	0	IDC	2

Table 2

Definitive surgery

Pt #	Date	Type	T	N	Stage
01	9/03/03	lumpectomy	T1	negative	I
02	10/16/03	mastectomy	T3	negative	II
03	11/03/03	mastectomy	T2	1/20	II
04	3/09/04	lumpectomy	T2	3/6	II
05	4/21/04	lumpectomy	T1	negative	I
06	8/23/04	mastectomy	T2	negative	II
07	11/08/04	mastectomy	T1	21/24	II
08	12/09/04	mastectomy	T1	2/34	II

Reportable Outcomes

There are no reportable outcomes of the study because of failure to accrue to target sample.

Conclusions

OSU 0125 showed it was feasible to collect fresh frozen and fixed breast cancer specimens at initial core biopsy and definitive surgery. This trial failed to meet its' accrual goals for following reasons:

1) In December 2004 the increased cardiovascular risks of celecoxib were made public. Many trials, including large cooperative group trials, were modified to eliminate the celecoxib portion. This resulted in a temporary halting of protocol accrual for about 6 months.

2) The primary and secondary endpoints were correlative studies measuring gene expression and biomarkers. Thus, it was not a therapeutic trial, and the surgeons in our group rapidly lost enthusiasm for this trial, especially with the potential cardiovascular risks associated with celecoxib.

3) Patients became aware of the increased cardiovascular risks of celecoxib either through the media or primary physicians. We had 2 patients refuse to enroll on trial specifically for the associated cardiovascular risks.

References

None

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

ACTION LETTER

DATE: December 20, 2004

FROM: JoAnne Zujewski, M.D., Clinical Investigations Branch, CTEP, DCTD, NCI

SUBJECT: Celecoxib Investigator Notification

TO: Investigators Using Celecoxib

The purpose of this letter is to alert investigators of the following results from a cardiovascular risk analysis of the Adenoma Prevention with Celecoxib (APC) and Prevention of Spontaneous Adenomatous Polyps (PreSAP) Trials by a special Cardiovascular Safety Committee (CSC) and relevant to celecoxib studies sponsored by the National Cancer Institute (NCI), Division of Cancer Treatment and Diagnosis (DCTD). A revision to the informed consent form and protocol is **required** by CTEP.

In light of recent reports that use of the selective COX-2 inhibitor, rofecoxib, is associated with an increased risk for experiencing cardiovascular problems, the Steering Committee of the Adenoma Prevention with Celecoxib (APC) Trial requested the assistance of a special Cardiovascular Safety Committee (CSC) to review its ongoing clinical trial of the related drug, celecoxib. The CSC has completed cardiovascular risk analysis for the APC Trial, as well as that of a related independent adenoma prevention trial, the Prevention of Spontaneous Adenomatous Polyps (PreSAP) trial. This second ongoing trial is similar to the APC Trial in its patient population, however involves a different schedule of celecoxib. The Steering Committees of both studies asked the CSC to assist each trial's existing independent Data Safety Monitoring Board (DSMB) in evaluating cardiovascular safety for their study patients.

The CSC found that patients randomized to celecoxib in the APC Trial had a statistically significant approximately 2.5 fold increase in their risk of experiencing a major fatal or nonfatal cardiovascular event compared to those taking placebo. This increased hazard was not observed in the patients taking celecoxib on the Pre-SAP Trial. On December 16, 2004, the CSC advised the DSMB of the APC Trial that continued exposure to celecoxib placed patients in this study at increased risk for serious cardiovascular events. Based upon this recommendation, the APC Trial DSMB has instructed investigators to immediately suspend study drug use for all patients currently on the study. These patients will remain under observation for the remainder of their planned study interval. For additional information, please go to the following URL: http://www.cancer.gov/newscenter/pressreleases/APCtrialCOX2.

Treatment studies sponsored by CTEP, NCI are in the attached list. Pending further follow-up and review of the toxicity information, we suggest that investigators consider whether or not to continue treatment with celecoxib. In view of these important clinical data, CTEP is requesting that ALL Principal Investigators of celecoxib protocols do the following:

1) Distribute this letter urgently to all Data Monitoring Committees (DMCs) and Data Safety and Monitoring Boards (DSMBs) with a copy of the e-mail or other rapid trackable communication (e.g. fax with return requested) to **Dr. Michael Montello** at PIO@CTEP.NCI.NIH.GOV within 5 working days of the date of this letter. Failure to comply within the 5-day timeframe may result in the temporary suspension of the principal investigator and enrollment of patients to the study.

2) Distribute this letter to all participating investigators and IRBs with a copy of the e-mail or other rapid trackable communication (e.g. fax with return requested) to **Dr. Michael Montello** at PIO@CTEP.NCI.NIH.GOV within 5 working days of the date of this letter. Failure to comply within the 5-day timeframe may result in the temporary suspension of the principal investigator and enrollment of patients to the study.

3) Amend protocols to include the results of the cardiovascular risk analysis and amend the informed consent documents to inform patients in lay terms of the results. Suggested informed consent language:

"Recently, an increased risk of heart attacks, strokes, and/or deaths resulting from heart or blood vessel disease has been reported among people taking celecoxib in clinical studies. Although the increased risk is 2 to 3 times greater than the risk of patients who did not take celecoxib, these serious adverse events are rare. Taking celecoxib may increase your risk of one of these events."

- For Cooperative Group studies, the revision to the protocol and informed consent form will be made by the Cooperative Group Operations office, forwarded to CTEP for approval, and circulated to the Group's investigators. Please follow any instructions provided by the Cooperative Group.

- For non-Cooperative Group studies, the principal investigator is required to forward a copy of the revised protocol and informed consent form to CTEP as outlined below.

4) Pending review/approval of the amended protocol and informed consent, accrual to studies may continue with approval of the DMC, DSMV, and IRB; however, prior to the local implementation of the amendment, potential study participants must be informed of these results, and this informed consent process documented in the patient's medical record/study chart.

5) All patients enrolled on protocols prior to implementation of this amendment should be informed of these results. At a minimum, patients should be verbally informed of this information, and the informed consent process should be documented in the patient's medical record/study chart.

6) **Within 5 working days, submit a plan for implementing the above actions to Dr. Michael Montello at** PIO@CTEP.NCI.NIH.GOV.

7) For Cooperative Group and Non-Cooperative Group Studies, submit all amendments to the protocol and informed consent form to NCI by 5:00 pm EST on **January 17, 2005**. The amendment cover letter must state "these amendments are in response to the memo from Dr. JoAnne Zujewski (zujewskj@mail.nih.gov, phone 301-496-2522) regarding the celecoxib cardiovascular risk analysis. Failure to comply within this timeframe may result in the temporary suspension of the principal investigator and enrollment to the study.

Please submit the amendment, the change memo, and the cover letter to Dr. Michael Montello at PIO@CTEP.NCI.NIH.GOV.

December 20, 2004

To All Investigators of Trials Using Celecoxib;

You were informed on 12/17/04 of the NCI's decision to suspend the use of celecoxib within the Adenoma Prevention with Celecoxib (APC) Trial 005 based on a recommendation from the Data Safety and Monitoring Committee. This decision was based on the data noted below (along with safety data from a separate adenoma prevention trial, Study 018 for comparison).

The following language is recommended for amending your trials' informed consents:

"Recently, an increased risk of heart attacks, strokes, and/or deaths resulting from heart or blood vessel disease has been reported among people taking celecoxib in clinical studies. Although the increased risk is 2 to 3 times greater than the risk of patients who did not take celecoxib, these serious adverse events are rare. Taking celecoxib may increase your risk of one of these events."

As ever, we will forward more information to you as it becomes available.

Leslie Ford, M.D. as
Associate Director for Clinical Research
Division of Cancer Prevention
National Cancer Institute

Absolute Number of Events

1. STUDY 005

Endpoint	Placebo N=679 n(%)	Celecoxib 200 mg PO BID N=685 n(%)	Celecoxib 400 mg PO BID N=671 n(%)	Celecoxib EITHER DOSE N=1356 n (%)
CV death, MI, stroke	6 (0.9)	15 (2.2)	20 (3.0)	35 (2.6)

2. STUDY 018

Endpoint	Placebo N=628 n(%)	Celecoxib 400 mg PO QD N=933 N(%)
CV death, MI, stroke	11 (1.8)	16 (1.7)

Relative Risk

1. STUDY 005

Endpoint	Celecoxib 200 mg PO BID N=685	Celecoxib 400 mg PO BID N=671	Celecoxib EITHER DOSE N=1356
CV death, MI, stroke	2.5 (1.0, 6.3)	3.4 (1.4, 8.3)	2.9 (1.2, 6.9)

2. STUDY 018

Endpoint	Celecoxib 400 mg PO QD N=933
CV death, MI, stroke	1.0 (0.5, 2.1)

Charles Shapiro, MD
James-Solove Cancer Center
Ohio State University
B421 Starling-Loving Hall
320 West 10th Avenue,
Columbus, OH 43210

Dear Dr. Shapiro,

Important information from the Independent Data Safety Monitoring Boards (IDSMB) for the Adenoma Prevention with Celebrex (APC) trial and the Prevention of Spontaneous Adenomatous Polyps (PreSAP) trial was received during the evening of December 16th.

As an Investigator in a trial where Celebrex is a component of therapy, we feel it is important to share the following information with you. Please see the attached press release.

APC Trial Rls Dec 17.pdf

Regards,
Pfizer Oncology Celebrex Team

IRB protocol # 2001C0266
OSU protocol # OSU 0125

THE EFFECT OF CELECOXIB (CELEBREX®) ON THE AROMATASE GENE (CYP19) EXPRESSION IN HUMAN BREAST CANCER

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Charles Shapiro, M.D.

Pathologist: Ping Wen, MD

Radiologist: John Olsen, MD

Statistician: Donn Young, PhD

Research Nurse: Marsha Hauger, RN

Revised: July 31, 2001
Revised: August 28, 2002
Revised: September 1, 2003
Revised: March 20, 2004
Revised: August 1, 2004
Revised: January 6, 2005
Revised: April 1, 2005

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1. OBJECTIVES

Primary:

To evaluate aromatase (CYP19) and estrogen receptor (ER) gene expression by reverse-transcriptase polymerase chain reaction (RT-PCR) in response to a selective cyclooxgenase-2 (COX-2) inhibitor, celecoxib (Celebrex®) in paired tumor tissue collected at the time of the initial diagnosis and at definitive surgery for localized non-metastatic breast cancer patients.

Secondary:

To evaluate the effect of celecoxib on the following biomarkers: estrogen receptor, progesterone receptor, Her2-neu, Ki-67, COX-1, COX-2, CYP19, CD31, and PGE_2, and aromatase activity in paired tissue specimens by standard immunohistochemical methods.

2. BACKGROUND/RATIONALE

Normal breast epithelium, and the sequential multi-step process that results in the transition from normal breast epithelium to breast cancer, is dependent on estrogens. Estrogens not only play an important role in the genesis of breast cancer, but understanding the action of estrogen is the basis of hormonal treatment of early stage and advanced breast cancer, and efforts to prevent breast cancer in high risk women. In addition to systemic production of estrogens, it has been recognized that estrogens are produced within the microenvironment of the breast tumors. The regulation of "local" estrogen production is based on a complex interactions between stromal tissue-tumor cells, that has increasingly been recognized as fundamental to process of breast cancer development and growth (1).

Aromatase (CYP19) Activity in Breast Cancer:

The P450 enzyme complex aromatase is the enzyme responsible for the conversion of androgen precursors to estrogen (2). Aromatase activity is greatest in the ovaries of premenopausal women, placenta of pregnant women, and the peripheral adipose tissue of postmenopausal women. More recently, aromatase activity has been demonstrated in normal breast tissue, and the highest levels in breast tumor tissue (3,4). In women with locally advanced breast cancer, anastrozole (Arimidex®), an aromatase inhibitor, was a potent suppressor of intratumoral estrogen levels (5). The gene responsible for aromatase activity is CYP19, and the regulation of CYP19 varies with tissue-specific promoters (6,7). In contrast to peripheral adipose tissue where the promoter is responsive to glucocorticoids and cytokines, in breast tissue there is a switch in the major promoter region (to promoter II) such that it is responsive to intracellular cAMP.

Cyclooxygenases (COX-1, 2) in Breast Cancer:

COX-1 and COX-2 enzymes are responsible for the conversion of arachidonic acid to prostaglandins. Prostaglandin E2 (PGE2) increases intracellular cAMP levels and stimulates estrogen biosynthesis (8). Investigators at Ohio State University first reported that the COX-2 gene was overexpressed in human breast cancers but not normal breast tissue (9) and that there is a strong correlation between the levels of expression of COX-1, COX-2, and CYP-19 in human breast

cancers (10). Investigators from Memorial Sloan Kettering Cancer Center demonstrated COX-2 is overexpressed in human breast cancers that overexpress HER-2/*neu*, whereas COX-2 was expressed at low levels in cancers that do not overexpress HER-2/*neu* (11). Taken together, these observations support the COX-2 enzyme as a potential therapeutic target in breast cancer.

COX-2 Inhibitors:

The selective COX-2 inhibitor, celecoxib (Celebrex®), is indicated for the treatment of osteoarthritis and rheumatoid arthritis. The hallmark of this drug is it is associated with less side effects than non-steroidal inflammatory drugs (NSAIDs), particularly gastroduodenal ulcers. Randomized trials demonstrate three to four-fold reduced incidence of gastroduodeneal ulcers relative to NSAIDs both for short and long term-administration.

COX-1, 2 Inhibition in Cancer:

NSAIDs and selective COX-2 inhibitors inhibit the growth of transplanted mammary tumors, 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary cancers, reduce colonic polyp formation and chemically-induced aberrant crypt formation, and are effective in prevention models of colon and breast cancer (12-16). Human epidemiologic studies show that there is there is a dose and duration-dependent relationship between NSAIDs and the risk of colon and breast cancer (16,17).

A placebo-controlled randomized trial on the efficacy of celecoxib in reducing the incidence of colorectal polyps patients with familial adenomatous polyposis (FAP) was recently reported (18). In that study, the doses were celecoxib 100 mg and 400 mg orally twice per day for 6 months. Both doses of celecoxib were well tolerated, without any suggestion of increased side effects at the higher dose. The most common toxicities were diarrhea (placebo 13%; 100mg 19%; and 400 mg 13%) and abdominal pain (placebo 13%; 100 mg 3%; and 400 mg 7%). Treatment with celecoxib 400 mg BID caused a 28% reduction in the number of colorectal polyps compared to a 4.5% reduction for placebo ($p=0.003$). Similarly, the total polyp burden (defined as the sum of polyp diameters) was significantly reduced ($p=0.001$) in patients receiving celecoxib 400 mg BID (30.7% reduction) compared to those receiving placebo (4.9% reduction). Furthermore, 53% of patients randomized to celecoxib 400 mg BID responded to treatment (ie: experienced at least a 25% reduction in polyp number), while only 7% of placebo treated patients responded similarly ($p=0.003$). These quantitative findings were supported by the results of the qualitative, global assessments of the endoscopic appearance of the GI tract in which significant improvement was noted for the colon, rectum, and duodenum in patients receiving celecoxib 400 mg BID ($p\leq0.033$ vs placebo). The corresponding changes in the group receiving celecoxib 100 mg BID did not reach statistical significance when compared to placebo. Based on the results of this study, celecoxib 400 mg BID was approved as adjunctive therapy for patients with FAP.

Hypothesis:

The observation that COX-1,2 expression is strongly correlated with CYP19 expression in human breast cancer leads to the following hypothesis: overexpression of COX-2 gene leads to increased levels of PGE2 (and intracellular cAMP) that activates the CYP19 promotor and increases gene expression. We hypothesize that celecoxib (Celebrex®), a selective COX-2 inhibitor, will decrease PGE2 in breast tumor tissue and will decrease gene expression of CYP19. The decreased expression of CYP19 will lead to a decrease in estrogen biosynthesis in breast tumor tissue. To test this

hypothesis, tumor tissue will be collected from breast cancer patients at the initial diagnosis of breast cancer and again at the definitive surgery (lumpectomy or mastectomy) according to the methods outlined in a completed Ohio State University (OSU) protocol (protocol # 9928 "Fresh Tissue Acquisition at the Initial Diagnosis and Definitive Surgery of Localized Breast Cancer Patients"). In the 10-14 day interval before the definitive surgery is performed, patients will receive celecoxib (Celebrex®). The tissue samples collected before and after treatment with the COX-2 inhibitor will be evaluated for gene expression of COX-2 and CYP19. If our hypothesis is correct, then expression of the CYP19 gene will decrease in response to celecoxib (Celebrex®). Furthermore, this study will provide preliminary data to a) support a mechanism whereby COX-2 inhibitors decrease estrogen production within breast tumors by decreasing CYP19 expression; and b) provide the rationale for initiating larger chemoprevention and therapeutic trials of COX-2 inhibitors in high risk and breast cancer patients.

Protocol 9928:

"Fresh Tissue Acquisition at the Initial Diagnosis and Definitive Surgery of Localized Breast Cancer Patients**"** was supported by a OSU Comprehensive Cancer Center Grant. The main objectives of protocol 9928 were to establish the feasibility of collecting fresh frozen tissue from with women at the initial diagnostic biopsy of breast cancer and then at the definitive therapeutic surgery, either lumpectomy or mastectomy. The Tissue Procurement Service of the OSU Medical Center (OSUMC) managed all tissue specimens. The protocol enrolled 17 women with localized (stage I-III) breast cancer and results are available in the first 14 matched pairs of breast cancer specimens. The matched pairs are analyzed for standard H&E staining, immunohistochemical staining estrogen receptor, progesterone receptor, HER2-neu overexpression (Hercept Test™), and the proliferation marker Ki-67. A single pathologist analyzed the tissue specimens and a second independent pathologist is reviewing the specimens to assess the degree of concordance. The results are described below.

Biopsy	Hist	ER	PR	HER2	Ki-67	Surg	Hist	ER	PR	HER2	Ki-67
1	IDC; HG	+	+	O	*	1	IDC; HG	+	+	O	< 10%
2	IDC; HG	+	*	1+	70%	2	IDC; HG	**	-	0-1+	40-90%
3	IDC; HG	-	-	1+	60-70%	3	IDC; HG	+*f*	+*f*	0	30-60%
4	IDC; HG	-	+	0	+*f*	4	IDC; HG	+	+*f*	1+	10%
5	IDC; HG	+	+	0	10%	5	IDC; HG	+	+	0	< 10%
6	IDC; HG	-	-	3+	40-80%	6	DCIS	-	+	3+	20-30%
7	IDC; HG	+	+	0	80%	7	IDC; HG	+	+	0	70%
8	IDC; IG	+	+	3+	20-25%	8	IDC; HG	+	+	*	20-25%
9	IDC; IG	+	-	2-3+	5-30%	9	IDC; IG	+	-	1-2+	10-20%
10	IDC; HG	-	-	1+	90%	10	IDC; HG	-	-	2+	80-95%
11	IDC; HG	-	+	3+	40-60%	11	IDC; HG	+	-	3+	10%
12	IDC; IHG	+	+	3+	30%	12	IDC; HG	+	+	2+	30-40%
13	IDC; IHG	+	+	1+	10%	13	IDC; IG	+	+	1+	10%
14	IDC; IG	+	+	0-1+	< 10%	14	IDC; IG	+	+	0-1+	< 5%

Abbreviations: invasive ductal cancer (IDC); high grade (HG); intermediate grade (IG)
*Not enough tumor tissue for semi-quantitation
**Control negative, test to be repeated
f Weakly positive

Protocol 9928 established the feasibility of collecting fresh frozen tissue specimens from women at initial diagnosis and definitive surgery without comprising the standard pathological evaluation required for patient care. In addition, it provides the methods of tissue collection that is described below.

3. PATIENT SELECTION

Inclusion criteria:

3.1 A woman with cytologically or histologically proven invasive breast cancer; either $T_{1\text{-}2\text{-}3}$, $N_{0\text{-}1}$, M_0. Women with bilateral breast cancers are eligible.

3.2 A women age 18 or older.

3.3 Tumor must be present following core needle biopsy as determined by physical exam or ultrasound.

3.4 A woman has no underlying medical condition that would prohibit surgical excision of her breast tumor.

3.5 A woman is fully informed about the nature of the study and gives consent.

Exclusion criteria:

3.6 A woman with a known history of aspirin or NSAID-induced asthma, urticaria, or allergic reactions.

3.7 A woman with locally advanced breast cancer as defined by clinical AJCC Stage IIIB disease.

3.8 A woman who has received a COX-2 inhibitor, or NSAID within 7 days of study drug.

3.9 A women who is taking fluconazole, or other drugs in the same class as fluconazole.

3.10 A woman who will receive chemotherapy or hormone therapy prior to surgical excision.

3.11 Women with only ductal carcinoma in-situ are excluded.

3.12 A women who is pregnant. Women of childbearing potential will be given a pregnancy test prior to study entry.

3.13 A woman with a previously documented history of allergy to sulfonamides severe enough in nature to require emergency room treatment or hospitalization.

3.14 A woman with a known history of myocardial infarction in the past 6 months.

4. PATIENT ENTRY

4.1 Women will be screened for eligibility for this trial by a research nurse or nurse practitioner working within the Comprehensive Breast Health Services (CBHS). In most cases, eligible women will be recruited by surgeons who work at the CBHS. The surgeon, research nurse, or nurse practitioner will provide the information about the study, possible risks of study, other options including not participating in the study, emphasize that participation is entirely voluntary and that if the woman chooses not to participate in the study her medical care will not be affected in any way, continue to answer all questions that arise, and assess how well the woman understands the aims of the study, risks, alternatives. Women who give their informed consent will be registered with the Clinical Trials Office.

5. TREATMENT PLAN

Thirty four women with highly suspicious breast masses identified by mammography, ultrasound, or physical examination will recruited at the Comprehensive Breast Health Services of Ohio State University (OSU). Prior to the diagnostic biopsy, patients will be asked to review and sign a screening consent form which pertains to the collection of 2-4 extra breast core biopsy samples at the time of the diagnostic breast biopsy. If the diagnostic biopsy indicates invasive breast cancer, and the patient enrolls on the protocol, the extra tissue cores will be used for research purposes related to treatment on this trial. If the patient does not participate in the protocol, the tissue cores will be destroyed and will not be used for research purposes.

Tissue will be obtained using standard techniques of core needle biopsy. For non-palpable lesions, core biopsy will be obtained under ultrasound guidance. Six core biopsies will be obtained using a spring loaded 14 gauge core biopsy device. Two core biopsy specimens will be formalin fixed for permanent histopathologic evaluation (Pathology cores) in the standard manner. The other 4 biopsy specimens will be used for research purposes. Two of the cores will be formalin fixed and used to perform immunohistochemical stains. The remaining two cores will be snap frozen and used to perform RNA studies. Following permanent histopathologic evaluation of the Pathology Cores, if further diagnostic tissue is needed (as determined by the reporting pathologist), then the formalin fixed tissue Research Cores will be used for diagnostic purposes by the pathologist. The OSU Tissue Procurement Service will maintain all tissue until it is felt that adequate histologic diagnostic material has been obtained.

Optimally, 4 extra cores for research purposes should be obtained at screening. However, if one or more extra cores are obtained, the woman would be able to enroll on the trial. If 4 cores to be used for research are not obtained in 12 biopsy attempts, the physician will stop the biopsy procedure. If the physician is not able to obtain extra core biopsy tissue to be used for research purposes, the woman will not be enrolled on the study.

Women enrolled on the study will receive 400 mg BID of celecoxib (Celebrex®) by mouth starting within 7 days after the biopsy and ending treatment the day before the definitive surgery (either lumpectomy or mastectomy). Patients will receive a minimum of 7 days celecoxib (Celebrex®) and maximum of 28 days. On average it is expected that they will receive celecoxib (Celebrex®) for 10 days. Participation in this study will not delay the definitive surgery. During the definitive surgery,

fixed and fresh frozen tissue will be collected and processed in a similar manner to the intial tissue. Tumor tissue that is acquired at the initial diagnosis and the definitive surgery will be evaluated by an OSU pathologist by standard H&E staining (i.e. histologic type, histologic grade, and lymphatic vessel invasion) as well as a variety of immunohistochemical stains for estrogen receptor, progesterone receptor, Ki-67, Factor VIII, HER2-neu, COX-1, and COX-2. In the fresh frozen core biopsies, tumor cells will be identified and collected using Laser Capture Microdissection (LCM). The LCM is available in the OSU Pathology Department and is staffed by a dedicated technician. RNA will be isolated from the tumor cells in paired samples and will be analyzed for CYP19 and COX-1, COX-2 gene expression by RT-PCR in the laboratory of Dr. Brueggemeier (OSU, Chairperson, Department of Medicinal Chemistry and Pharmacology, College of Pharmacy) according to published methods. Additionally, PGE_2 and aromatase enzyme activity will be measured according to published methods.

6. AGENT FORMULATION AND PROCUREMENT

6.1 Drug: Celecoxib (Celebrex®)

6.2 Classification: Cyclooxgenase-2 Inhibitor

6.3 Molecular Formula: $C_{17}H_{14}F_3N_3O_2S$ M.W. 381.38

6.4 Product description: Celecoxib (Celebrex®) 200 mg oral capsules

6.5 Storage requirements: Store at room temperature (59-86° F)

6.6 Stability: Stable at room temperature.

6.7 Route of Administration: The drug is to be given orally twice per day.

6.8 Availability: Celecoxib (Celebrex®) is supplied by Pfizer.

6.9 Agent Ordering: The research pharmacist or study nurse will be responsible for contacting Pfizer to order celecoxib (Celebrex®).

6.10 Agent Accountability: The research pharmacist at the Comprehensive Breast Health Services will be responsible for dispensing and storing Celecoxib (Celebrex®) in the pharmacy. Drug will stored in a secured location within the pharmacy and dispensed to women enrolled on study.

7. EXPECTED TOXICITY

Toxicity will be graded on CTC scale Version 2.0

7.1 Celecoxib (Celebrex®) is commercially available and approved for the relief of signs and symptoms of osteroarthritis and rheumatoid arthritis.

7.2 Expected toxicity: The following adverse experiences occurred in 2% or more of celecoxib (Celebrex®) patients relative to placebo and ibuprofen:

	Placebo (n=1684)	Percent celecoxib (Celebrex®) 100 or 200 mg BID (n=4146)	Ibuprofen 2400 mg/day (n=387)
Nausea	4.2	3.5	3.4
Dyspepsia	6.2	8.8	10.9
Diarrhea	3.8	5.6	9.3
Abdominal pain	2.8	4.1	9.0
Headache	15.8	20.2	15.5
Peripheral edema	1.1	3.7	3.8
Dizziness	1.7	2.0	1.3
Sinusitis	4.3	5.0	5.4
Upper respiratory tract infection	6.7	8.1	9.8
Back pain	3.6	2.8	2.6
Gastroduodenal ulcers	2.0-2.3	1.5-4.1	9.6

*12 weeks in duration

In a study of 77 patients who were randomly allocated 100 mg po BID, 400 mg po BID, or placebo there were no significant differences in side effects between celecoxib (Celebrex®) and placebo treatment groups (18).

Concerns have been raised about selective COX-2 inhibitors and the risk of thrombotic events including myocardial infarction. Results of a recent meta-analysis show a small increased rate of myocardial infarction for celecoxib (Celebrex®) (0.80%) versus placebo (0.52%), p=0.02[18a]. Limitations of this meta-analysis included the use of healthy historical controls and evaluating the rates of myocardial infarction in these randomized studies was an unplanned, post hoc analysis. In a large individual study, that also permitted some patients to receive aspirin, the incidence of cardiovascular events in patients receiving either celecoxib or other non-steroidal anti-inflammatory agents was not increased. However, there was an increase in serious gastrointestinal side effects among those celecoxib and aspirin.

In December 2004, important safety information regarding celecoxib was reported from the Independent Data Safety Monitoring Boards (IDSMB) of the US National Cancer Institute monitoring the Adenoma Prevention with Celebrex (APC) and the Prevention of Spontaneous Adenomatous Polyps (PreSAP) trials. In addition, results from a third long term celecoxib study, a US National Institutes of Aging Alzheimer's Prevention study (ADAPT), were also recently reported.

The cancer prevention studies used the same cardiovascular review board (commissioned by the data safety monitoring boards of the two respective trials) to adjudicate the results and used the same analysis methods. Patients in the studies were treated for up to 4 years. Cardiovascular risk analysis performed on the Adenoma Prevention with Celecoxib (APC) and Prevention of Spontaneous Adenomatous Polyps (PreSAP) trials showed there was a 2-3-fold higher risk of cardiovascular events in patients receiving Celebrex 200 mg twice per day (15/685=2.2%) and 400 mg twice per day (20/671=3.0%) versus placebo (6/679=0.9%).

A third trial (ADAPT) compared Celebrex to either naproxen sodium or placebo in a group of patients at risk for Alzheimer's disease treated for up to 3 years. Preliminary safety results (not yet adjudicated) from that study indicate an increased cardiovascular risk with naproxen sodium but not celecoxib relative to placebo.

In view of this information, we have excluded women with a myocardial infarction or other thrombotic event.

Sulfonamide Allergy

Sulfonamides are generally divided into 2 groups: 1) aromatic amines, including sulfonamide antibacterials, and 2) nonaromatic amines, including Celebrex. It is the aromatic amine group at the N4 position that is thought to be critical in the development of serious hypersensitivity reactions. Generally when patients report a sulfonamide allergy, they are referring to an allergy to sulfonamide antibacterials rather than nonaromatic amine drugs (19). Some researchers argue a lack of documentation to support cross-reactivity (allergic reaction) between sulfonamide antibacterials and other sulfonamide medications such as Celebrex (20).

A meta-analyses of data collected from 14 separate Celebrex trials on over 11,000 patients with a history of sulfonamide hypersensitivity demonstrated that the incidence of subsequent allergic reactions to Celebrex were not statistically significant when compared with placebo or active comparator (other NSAIDs) (21). Results of a controlled British study suggested that in a patient with a history of sulfonamide antibiotic allergy, a subsequent allergic reaction to a sulfonamide nonantibiotic appears to be due to a predisposition to allergic reactions in general, rather then to cross-reactivity with sulfonamide-based drugs (22).

Patients will be evaluated for a prior history of allergic reactions including sulfonamide allergy. Patients will be assessed throughout the study treatment for signs and symptoms of an allergic reaction. The signs and symptoms of an allergic reaction include, but are not limited to, skin rash, hives, skin disorders, abdominal pain, and shortness of breath. The decision on whether or not to stop the Celebrex will be at the discretion of the treating physician. Patients with a documented prior history of severe allergic reaction to sulfonamides that required emergency room treatment or hospitalization will be excluded from the study.

7.3 Potential Drug Interactions: Metabolism of celecoxib (Celebrex®) is via the P450 2C9 enzyme and co-administration of drugs that are known to inhibit 2C9 should be done with caution. *In vitro* studies suggest that celecoxib is an inhibitor of the P450 2D6 enzyme as well and there is potential for drug interactions *in vivo* with drugs that are metabolized by this enzyme. The following describes possible drug interactions:

7.3.1 ACE-inhibitors: NSAIDs may diminish the antihypertensive effect of ACE-inhibitors. Blood pressure will be monitored weekly during study for woman on ACE-inhibitors.

7.3.2 Furosemide: NSAIDs can reduce the natriuretic effect of the furosemide and thiazdes in some patients. Blood pressure will be monitored weekly during study for woman on ACE-inhibitors.

7.3.3 Aspirin: The rate of gastrointestinal ulcers may increase with concomitant use of celecoxib (Celebrex®) and aspirin. Woman may not take aspirin during this study.

7.3.4 Fluconazole: Concomitant administration of celecoxib (Celebrex®) and fluconazole results a two-fold level increase in celecoxib (Celebrex®) levels. Woman may not take fluconazole, or other drugs in the same class as fluconazole, during this study.

7.3.5 Lithium: Concomitant administration of celecoxib (Celebrex®) and lithium results in an increase in steady-state lithium levels. Woman on lithium will have drug levels monitored weekly during study.

7.3.6 Coumadin: Although celecoxib (Celebrex®) does not alter PT/INR in patients on coumadin, there have been reports of bleeding in elderly patients taking both coumadin and celecoxib (Celebrex®). Woman on coumadin will have their PT/INR measured weekly during study.

8. CORRELATIVE STUDIES

Real Time PCR for CYP19, COX-1, COX-2, and ER gene expression:

Reverse transcription will be performed on breast tissue samples in a final volume of 20 μL containing 1 μg total RNA, 10 mM of DTT, 100 ng of Random Primers; 250 mM Tris-HCl, pH 8.3, 37 5mM KCl, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 5 units of RNase inhibitor, and 200 units of Superscript RNase H Reverse Transcriptase (Gibco BRL). The reaction is incubated at 23° C for 15 min., 42° C for 50 min., and 95° C for 5 min. A negative RT control is prepared using patient RNA but without the addition of reverse transcriptase in order to check for contaminating genomic DNA in the RNA sample. To terminate the reaction, samples are then incubated for 5 min. at 99°C.

The resulting cDNA from reverse tranciption will be used for PCR amplification of the indicated targets using Taqman technology with the Applied Biosystems Prism 7700 thermocycler. Primers for PCR amplification are selected from two exons separated by one or more long intronic sequences, which allow identification of amplification of contaminating genomic DNA. Probes will be labeled with 6-FAM on the 5' end and TAMRA on the 3' end. The primers and probe to be used for CYP19 are:

CYP19 primer: 5'-TGTCTCTTTGTTCTTCATGCTATTTCTC-3'
CTP19 reverse primer: 5'-TCACCAATAACAGTCTGGATTTCC-3'
CYP19 probe: 5'-TGCAAAGCACCCTAATGTTGAAGAGGCAAT-3'

CYP19 gene expression will be normalized to the 18S ribosomal RNA internal control gene. A 4 microliter volume of cDNA will be used in a 50 ul reaction volume containing Taqman Universal Buffer, 400 nM each of sense and antisense primers, and 200 nM of target-specific probe. A negative control water and probe only sample will be used to correct for background fluorescence. All samples will be heated at 50°C for 2 minutes and followed by 95°C for 10 minutes to activate enzymatic activity. A total of 45 sequential cycles at 95°C, 15 seconds, and 60°C, 1 minute will be used to obtain a saturating fluorescence signal. An arbitrary threshold (CT) set in the linear range of each sample will be used to quantify relative abundance of amplicon product. Normalization to internal control 18S RNA will yield fold difference results by the following formula

$$\Delta\Delta CT = \Delta CT(\text{experimental}) - \Delta CT(\text{control}) = |n|$$

$$\text{Fold} = 2^{|n|}$$

A subsequent 3% TAE-agarose gel will be used to confirm all fluorescence generated by a single amplified product.

Real-time PCR for COX-1 and COX-2 will be carried our as described above using the following primers and probes:

COX-1 primer: 5'-ATGACAGGGCAGAGCAGGAA-3'
COX-1 reverse primer: 5'-TTGGACCACGGCTGCAG-3'
COX-1 probe: 5'-ACAGGAAGCTGGCAGAACGGAGGAG-3'

COX-2 primer: 5'-GAATCATTCACCAGGCAAATTG-3'
COX-2 reverse primer: 5'-TCTGTACTGCGGGTGGAACA-3'
COX-2 probe: 5'-TGGCAGGGTTGCTGGTGGTAGGA-3'

Real-Time PCR for ERα will be carried out as described above using the following primers and probe:

ERα forward primer: 5'-AGCTCCTCCTCATCCTCTCC-3'
ERα reverse primer: 5'-TCTCCAGCAGCAGGTCATAG-3'
ERα probe: 5'-TCAGGCACATGAGTAACAAAGGCA-3'

Measurement of PGE_2 Biosynthesis: Slices of patient tissue will be incubated in DMEM media. The amount of PGE_2 released into the culture media will be determined using an [^{125}I]- PGE_2 radioimmunoassay kit (DuPont NEN) or an enzyme immunoassay kit (Cayman Chemical). At the end of an experiment, aliquots of the media will be removed and processed following the procedures in the RIA or EIA kit. Each kit uses antiserum that is highly sensitive and specific for PGE_2, with an iodinated analog as the radiotracer in RIA or a coupled enzyme (alkaline phosphatase or acethylcholinesterase) in the EIA kit. Standard curves will be prepared ranging from 1.0 pg/tube to 100 pg/tube, and the lower level of detection is 0.44pg/ml. The expected levels of released PGE_2 into the media in a T-25 flask should range from 1.0 to 10 pg/ml media. Enzymatic activity can also be determined from cell lysates by measuring the conversion of arachidonic acid (100 μM) to PGE_2 at 37°C for 30 minutes.

Measurement of Aromatase Activity: Aromatase activity in the patient tissue slices will be determined by measuring the conversion of [1β-^{3}H]-androstenedione to ^3H_2O and unlabeled estrone. This radiometric asay method in MCF-7 cells gave equivalent results to those obtained with the assay methods employing product estrogen isolation. [1β-^{3}H]-Androstenedione (30 nM, 2 μCi) will be dissolved in 10 μl 95% ethanol and added to the cultures. At 8 hours, media is removed, a solution of col 30% TCA (10 ml) is added to the media to precipitate proteins, and the media extracted three times with $CHCI_3$ (30 ml each time). An aliquot (2.0 ml) of the aqueous media layer will be then treated with 0.66% dextran-coated charcoal, followed by centrifugation at 5000 x g., to remove any residual ^{3}H-steroid. The aqueous solution will then be added to Formula 963 (8.0 ml) and the gel counted by LSC to determine the amount of radioactivity.

9. STUDY CALENDAR

	Before Study#	Weekly	Before Surgery
CBCD, PLTs, INR**	X	X**	X
Creatinine	X		X
Blood Pressure*	X	X*	X
Lithium Level+	X+	X+	X+
Serum pregnancy test	X		
Toxicity/allergic reaction assessment		X++	
Duration of celecoxib 400 mg po BID		Minimum of 7 days; maximum of 28 days	

Within 1 week of study drug

* Weekly blood pressure is required only for women on ACE-inhibitors

**The INR is required only for women on coumadin

+ Lithium levels are required only for women on Lithium

++ Patients with a prior history of allergic drug reaction, including sulfonamide allergy, should be contacted within 2-3 days after starting Celebrex, at the 1 week time point, and at each study visit, to assess for signs and symptoms of allergic reaction to Celebrex.

10. REGULATORY AND REPORTING REQUIREMENT

10.1 Assessment of Safety: In the event of an adverse event, the first concern will be for the safety of the subject. Investigators are required to report to any SERIOUS adverse event that may be expected or unexpected, as defined below, and reasonably or probably regarded as caused by drug occurring within a protocol-defined period of treatment and post-treatment follow-up.

10.2 Adverse Event Definition: An adverse event is any untoward medical occurrence in a patient or clinical investigation subject administered a drug regardless of causality assessment. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory

finding), symptom or disease temporally associated with the use of an investigational product, whether or not related to the investigational product.

Adverse events that are both serious and unexpected must be reported to the IRB within 10 working days utilizing the IRB SAE report form. Deaths should be reported to the IRB within 3 working days. All serious adverse events (unexpected and/or expected) that occur at the Ohio State University are to be reported to the IRB through the annual continuing review process.

Adverse events that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (301-619-2165; non-duty hours call 301-619-2165 and send information by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. Address the report to: U.S. Army Medical Research and Material Command, ATTN: MCMR_RCQ, 504 Scott Street, Fort Derick, Maryland 21702-5012.

Any adverse events related to the study will be communicated by the principal investigator to the treating physician responsible for study participant's care.

All amendments that relate to this study will require approval by the OSU IRB as well as the HSRBB.

11. STATISTICAL CONSIDERATIONS

11.1 The primary objective is to determine if a minimum of 7 and estimated average of 10 days of 400 mg BID celecoxib (Celebrex®) results in changes in aromatase (CYP19) and estrogen receptor (ER) gene expression in tumor tissue collected at the time of the initial diagnosis as compared with tumor resected at the time of definitive surgery. Previous studies (10) demonstrate that the relative quantitative levels of CYP19/36B4 show a mean of 0.36 with a standard deviation of 0.21 [n = 19]. Similarly, COX-1 and COX-2 expression normalized to HPRT show means ± standard deviations of 0.91 ± 0.68 [n = 22] and 0.84 ± 0.88 [n = 22], respectively. Using a paired t-test, a sample size of 34 patients will provide 80% power to detect an effect size of 0.50 with a 0.05 two-sided level of significance. Since we do not have prior data on the variation inherent in serial samples for gene expression, we assume that the effect size of 0.5 errs on the conservative side and would easily allow us to determine if celecoxib (Celebrex®) results in a 50% decrease in the levels of gene expression. With the previous significant linear relationship between CYP19 and the sum of COX-1 and COX-2 expression in a smaller sample [$r = 0.80$, $P < 0.0001$, $n = 21$] we expect to provide a better estimate of this relationship with our larger sample size.

11.2 Minority Considerations

This protocol will be open to all eligible patients, regardless of ethnic origin. American Cancer Society data on the incidence of breast cancer demonstrate no significant differences between black and white women. Breast cancer also appears to be less common in Native American, Asian and Pacific Islander women than in whites based on 1996 estimates by the American Cancer Society. Nevertheless, SEER data show that the 5-year survival of black women with breast cancer [69%] is significantly [$P < 0.05$] less as compared to white women [84%] during the period 1986-1991. While we recognize differences in mortality based on ethnicity of patients with breast cancer, we will expect to accrue minority patients to this study in proportion to percentage of minorities that are represented in the population of patients served by the James.

Patients estimated to be enrolled on study:

	White, not of Hispanic Origin	Hispanic	Black, not of Hispanic Origin	Native Hawaiian or other Pacific Islander	Asian	American Indian or Alaskan Native	Total
Male	-	-	--	-	-	-	0
Female	28	1	4	0	1	0	34
Total	28 82%	1 3%	4 12%	0 0%	1 3%	0 0%	34 100.0%

12. STUDY PERSONNEL

12.1 Principal Investigator: Stephen Povoski, M.D., Associate Professor of Surgery, will be responsible for the reviewing eligibility, toxicity monitoring, ensuring that proper procedures of adverse reporting are followed, compliance is maintained with the OSU IRB and HSRRB, and will supervise every aspect of the study.

12.2 Co-investigators: Robert Brueggemeier PhD. Dr. Brueggemeier is Professor of Medicinal Chemistry, will be responsible for all the correlative science studies associated with this protocol. Charles L. Shapiro MD., Associate Professor of Medicine and Director of Breast Medical Oncology, will serve as a co-investigator for the study.

12.3 Pathologist: Ping Wen, MD, Professor of Pathology, will be responsible for all aspects of breast pathology associated with this protocol.

12.4 Radiologist: John Olsen MD, Professor of Radiology, will be responsible for all aspects of breast radiology associated with this protocol.

12.5 Research Nurse: Marsha Hauger, RN. The research nurse will coordinate all aspects of this study.

12.6 Medical Monitor: Miguel Villalona MD, Associate Professor of Medicine will serve as medical monitor. He is not associated with this particular protocol. He has agreed to provide medical care to research subjects for conditions that may arise during the conduct of the study, and monitor the subjects during the conduct of the study. In addition, he will review all serious and unexpected adverse events associated with the protocol, an outcome of the AE and the relationship of the AE to the study, provide an unbiased written report of the event within ten (10) calendar days of the initial report. He will also indicate whether he concurs with the details of the report provided by the study investigator.

13. STORAGE OF DATA and PATIENT CONFIDENTIALITY

13.1 The OSU Tissue Procurement Service will maintain all tissues obtained from participants in this study. Standard procedure of the Tissue Procurement Service is to remove all patient identifiers and assign a coded numbers for all samples. The results of studies performed on these coded samples will be stored in a computerized database. Access to the database will be restricted to the principal investigators, data managers, and the research nurse only.

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